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The purpose of this project is to generate monoclonal antibodies to receptor molecules for the angiogenic growth factor VEGF. These antibodies will be tested for their potential as inhibitors of VEGF-stimulated angiogenesis, which is thought to play an important role in the development of breast cancer. A number of hybridomas have been screened for neutralizing activity, but no confirmed positives have yet been identified. Modifications to immunization and screening protocols are being made. Research demonstrating the direct binding of the intracellular signaling enzyme PI 3-kinase to the Flt-1 receptor is in press.

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Introduction

The survival and growth of tumors is dependent on the neo-vascularization of the growing tumor mass. Prior to the vascularization of a tumor, a subpopulation of tumor cells acquire an angiogenic phenotype characterized by the production of polypeptide growth factors that stimulate endothelial cell proliferation, migration and differentiation. By activating cell surface receptor molecules, these tumor-derived angiogenic factors induce the growth of blood vessels into the tumor from pre-existing blood vessels. Thus, the process of tumor angiogenesis represents an attractive target distinct from tumor cells themselves for the development of new cancer therapeutic agents. Experimental therapies directed against various components of the angiogenic process including growth factors, receptor kinase activities, intracellular signaling enzymes, extracellular matrix molecules, and proteases are currently being evaluated. We are characterizing the signaling pathways utilized by vascular endothelial growth factor (VEGF), an angiogenic factor that is widely expressed by breast tumors, and we are generating neutralizing monoclonal antibodies to receptor molecules for VEGF. We will test the antibodies for the ability to block VEGF-induced endothelial cell proliferation and capillary growth in vitro. VEGF is known to play a crucial role in both normal embryonic development and in the malignant transformation of a number of different tissues.

Body of Report

Grant DAMD17-98-1-8348 was transferred at the end of July 2000 to the American Type Culture Collection (ATCC) where the PI occupies a laboratory on the Prince William campus of George Mason University. A one year extension to August 2002 was granted in July 2001. In June of 2001 the PI received approval from the Prince William Hospital IRB for a human subjects protocol, which was originally submitted in December 1999. This protocol is currently being reviewed by USAMRMC.

Specific Aim 1: Tasks 1-5

Vascular endothelial cell growth factor (VEGF) stimulates angiogenesis and human endothelial cell proliferation by binding to two cell surface receptor tyrosine kinase molecules: Flt-1 (fms-like tyrosine kinase) and KDR (kinase insert domain-containing receptor) (1,2). The affinity of Flt-1 for VEGF is about an order of magnitude higher than that of KDR. In recent years the ligand-binding regions of these receptors have been localized to the N-terminal three or four Ig-like loops in the extracellular domains (3-7). The second Ig-loop of Flt-1 is the primary determinant of ligand binding (3), and we have shown that either loop 1 or 3 is required in conjunction with loop 2 for high affinity VEGF binding (6). Both VEGF receptor types are essential for normal embryonic development as the targeted deletion of each receptor is embryonic lethal (8,9). In adult mammals both VEGF receptors are found predominantly on endothelial cells, however, KDR is also an early marker in hematopoietic cell development and is present on hematopoietic stem cells in bone marrow (10).

Because cytotoxic antibodies to KDR may have the potential to kill hematopoietic stem cells, we are focusing on raising monoclonal antibodies to Flt-1. We have created baculoviruses harboring cDNAs encoding full-length and truncated human Flt-1 receptors (6). These receptor constructs are expressed in the High 5 T. ni cell line obtained from Invitrogen. In addition, we have transfected High 5 cells with full-length receptor cDNA that is constitutively expressed. These sources provide the human Flt-1 molecules that are used to immunize mice. We have gone through one cycle of antibody production and analysis in which monoclonal antibodies were generated from spleens of mice immunized with High 5 cells expressing full-length Flt-1. Over 500 hybridoma supernatants were assayed for the ability to inhibit VEGF binding to High 5 cells expressing Flt-1. Thirty hybridomas whose supernatants moderately inhibited VEGF binding (40-60%) were subcloned, however, none of the hybridoma clones were positive on re-screening. Further hybridomas that were generated with splenocytes from mice immunized with full-length Flt-1 will be analyzed for neutralizing activity. In addition, mice will be immunized with insect cells expressing the three Ig-loop binding region of

Flt-1 to increase the probability of producing neutralizing antibodies. We are purifying the three loop Flt-1 construct from insect cells for use as an immunogen but have not yet achieved homogenous preparations. To increase the throughput of the antibody screen, we are developing an assay for cell-bound VEGF that utilizes streptavidin-conjugated horseradish peroxidase and enhanced chemiluminescence. This will allow the assay to be done in a 96-well dot blot format with fewer target cells.

The strategy of inhibiting the angiogenic activity of VEGF at the level of ligand-receptor interactions at the cell surface rather than at the level of intracellular signaling intermediates is supported by studies of intracellular pathways activated in endothelial cells in response to VEGF (11-13 and Appendix 1). The mitogen-activated protein (MAP) kinases ERKs 1 and 2, and p38 MAP kinase but not Jun N-terminal kinase (JNK) and phosphatidylinositol 3'-kinase (PI 3-kinase) were activated in response to VEGF. By using specific inhibitors of these kinases, we showed that activation of ERKs 1 and 2 and PI 3-kinase were required for VEGF-stimulated endothelial cell to proliferation (11). However, these pathways were not uniquely activated by VEGF but were also activated by epidermal growth factor and fibroblast growth factor-2. This observation of convergent growth factor signaling in endothelial cells indicates that therapeutic strategies aimed at inhibiting intracellular signaling components will not be specific for responses to VEGF. This conclusion is a compelling argument for focusing on proximal steps in VEGF signaling to inhibit angiogenesis.

We have extended our study on VEGF signaling in endothelial cells to show that PI 3-kinase, an enzyme whose activity is required for a mitogenic response to VEGF, binds directly to the Flt-1 receptor for VEGF (14, Appendix 2). This direct interaction is significant in that it implies that PI 3-kinase is activated through VEGF binding to Flt-1. To date PI 3-kinase activation has only been experimentally linked to the KDR receptor (15). Thus, it is likely that dual receptors signals are involved in PI 3-kinase activation.

Specific Aim 2: Tasks-6-8

The activities of antibodies specific for human receptor molecules cannot initially be characterized in vivo. The goal of this specific aim is to develop an in vitro microvessel growth assay using fragments of human vascular tissue obtained from umbilical cords. As reported last year, we have done pilot experiments indicating we have culture conditions that should allow endothelial cell outgrowth and microvessel formation from pieces of umbilical cord. Tissue fragments were plated on a collagen-coated substratum in DME/F12 nutrient medium supplemented with either 10% fetal bovine serum, 10 ng/ml fibroblast growth factor-2 (FGF-2), 10 ng/ml VEGF or FGF-2 and VEGF. After a growth phase in which cells migrated out of the tissue fragments and proliferated on the substratum, the nutrient medium was changed to one with a low calcium concentration. A large percentage of cells died in the new medium consistent with the inability of

fibroblasts to grow under conditions of low calcium (16). The remaining cells had the morphological characteristics of endothelial cells. Further development of this assay has been put on hold pending the approval of a human subjects protocol for the acquisition and research use of umbilical cord tissue. A protocol and consent form submitted in December 1999 to the Prince William Hospital in Manassas, VA were approved by the hospital's IRB in June 2001 after several revisions (see Appendix 3). The protocol and consent form are now being reviewed by a human subjects specialist at the USAMRMC.

Specific Aim 3: Task 9

To reduce the immunogenicity of mouse monoclonal antibodies in humans it has become standard practice to clone the complementarity determining regions of the antibody light and heavy chains into human antibody heavy and light chain cDNAs (17). A chimeric or humanized antibody is then expressed from the cDNAs in a mammalian host cell line. In anticipation of humanizing mouse monoclonal anti-Flt-1 antibodies, we have used mixed synthetic oligonucleotide primers (18) to sequence by RT-PCR the Ig heavy and light chain variable regions of a monoclonal antibody to the human EGF receptor (19). The same primer sets will be used to sequence anti-Flt-1 antibody variable regions.

Key Research Accomplishments

- Identification of a major autophosphorylation site on the Flt-1 VEGF receptor and direct demonstration of phosphotyrosine-dependent PI 3-kinase binding
- Cloning and sequencing of antibody heavy and light chain variable regions

Reportable Outcomes

Publications

1. Sato, J.D. and Yu, Y. (2000) VEGF signaling pathways in endothelial cell mitogenesis. DoD Breast Cancer Research Program Era of Hope Meeting. Atlanta, GA, June 8-12, p. 568.

2. Yu, Y., Hulmes, J.D., Herley, M.T., Whitney, R.G., Crabb, J.W., and Sato, J.D. (2001) Direct identification of a major autophosphorylation site on vascular endothelial growth factor receptor Flt-1 that mediate phosphatidylinositol 3′-kinase binding. Biochem. J. In press.

3. Sato. J.D. (2000) Hybridoma cultures for production of antibodies. Purification and specificity of antibodies. In: Protocols for Neural Cell Cultures, (S. Fedoroff and A. Richardson, eds.), Human Press, New Jersey.

4. Sato, J.D., Barnes, D.W., Hayashi, I., Hayashi, J., Hoshi, H., Kawamoto, T., McKeehan, W.L., Matsuda, R., Matsuzaki, K., Okamoto, T., Serrero, G., Sussman, D.J., and Kan, M. (2001) Specific cell types and their requirements. In: Basic Cell Culture: a Practical Approach, (J.M. Davis, ed.), Oxford University Press, Oxford. In press.

Conclusions

Several hundred hybridoma supernatants have been screened for neutralizing activity towards Flt-1, but no confirmed postives were identified. Immunization and screening procedures will be modified for future hybridoma fusions. The major conclusion of the research done thus far is that the convergence or overlap of signaling pathways initiated by different growth factors indicate therapeutic interventions of angiogenesis or other growth factor-induced processes should target early events in signaling cascades in order to achieve the greatest degree of specificity.

References

1. deVries, C., Escobedo, J., Ueno, H., Houck, K., Ferrara, N., and Williams, L.T. (1992) The fms-like tyrosine kinase, a receptor for vascular endothelial cell growth factor. Science 255: 989-991.

2. Terman, B.I., Dougher-Vermazen, M., Carrion, M.E., Dimitrov, D., Armellino, D.C., Gospodarowicz, D., and Bohlen, P. (1992) Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem. Biophys. Res. Commun. 187: 1579-1586.

3. Davis-Smyth, T., Chen, H., Park, J., Presta, L.G., and Ferrara, N. (1996) The second immunoglobulin-like domain of the VEGF tyrosine kinase receptor Flt-1 determines ligand binding and may initiate a signal transduction cascade. EMBO J. 15: 4919-4927.

4. Barleon, B., Totzke, F., Herzog, C., Blanke, S., Kremmer, E., Siemeister, G., Marme, D., and Martiny-Baron, G. (1997) Mapping the sites for ligand binding

and receptor dimerization at the extracellular domain of the vascular endothelial growth factor receptor Flt-1. J. Biol. Chem. 272: 10382-10388.

5. Cunningham, S.A., Stephan, C.C., Arrate, M.P., Ayer, K.G., and Brock, T.A. (1997) Identification of the extracellular domains of Flt-1 that mediate ligand interactions. Biochem. Biophys. Res. Commun. 231: 596-599.

6. Herley, M.T., Yu, Y., Whitney, R.G., and Sato, J.D. (1999) Characterization of the VEGF binding site on the Flt-1 receptor. Biochem. Biophys. Res. Commun. 262: 731-738.

7. Fuh, G., Li, B., Crowley, C., Cunningham, B. and Wells, J.A. (1998) Requirements for binding and signaling of the kinase domain receptor for vascular endothelial growth factor. J. Biol. Chem. 273: 11197-11204.

8. Fong, G-H., Rossant, J., Gertsenstein, M., and Breitman, M.L. (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376: 66-70.

9. Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X-F., Breitman, M.L., and Schuh, A.C. (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice.Nature 376: 62-66.

10. Risau, W. (1997) Mechanisms of angiogenesis. Nature 386: 671-674.

11. Yu, Y. and Sato, J.D. (1999) MAP kinases, phosphatidylinositol 3'-kinase, and p70 S6 kinase mediate the mitogenic response of human endothelial cells to vascular endothelial growth factor. J. Cell. Physiol. 178: 235-246.

12. Wu, L-W., Mayo, L.D., Dunbar, J.D., Kessler, K.M., Baerwald, M.R., Jaffe, E.A., Wang, D., Warren, R.S., and Donner, D.B. (2000) Utilization of distinct signaling pathways by receptors for vascular endothelial cell growth factor and other mitogens in the induction of endothelial cell proliferation. J. Biol. Chem. 275: 5096-5103.

13. Gille, H., Kowalski, J., Li, B., LeCouter, J., Moffat, B., Zioncheck, T.F., Pelletier,N., Ferrara, N. (2001) Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. J. Biol. Chem. 276: 3222-3230.

14. Yu, Y., Hulmes, J.D., Herley, M.T., Whitney, R.G., Crabb, J.W., and Sato, J.D. (2001) Direct identification of a major autophosphorylation site on vascular endothelial growth factor receptor Flt-1 that mediate phosphatidylinositol 3'-kinase binding. Biochem. J. In press.

15. Gerber, H-P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B.A., Dixit, V., and Ferrera, N. (1998) Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. J. Biol. Chem. 273: 30336-30343.

16. Sato, J.D., Hayashi, I., Hayashi, J., Hoshi, H., Kawamoto, T., McKeehan, W.L., Matsuda, R., Matsuzaki, K., Mills, K.H.G., Okamoto, T., Serrero, G., Sussman, D.J., and Kan, M. (1994) Specific cell types and their requirements. In: Basic Cell Culture: a Practical Approach, J.M. Davis, ed., Oxford University Press, Oxford.

17. Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S., and Winter, G. (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature 321: 522-525.

18. Orlandi, R., Gussow, D.H., Jones, P.T., and Winter, G. (1989) Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA 86: 3833-3837.

19. Wu, D., Wang, L., Sato, G.H., West, K.A., Harris, W.R., Crabb, J.W., and Sato, J.D. (1989) Human epidermal growth factor (EGF) receptor sequence recognized by EGF-competitive monoclonal antibodies. Evidence for the localization of the EGF-binding site. J. Biol. Chem. 264: 17469-17475.

Appendices

Appendix 1

Sato, J.D. and Yu, Y. (2000) VEGF signaling pathways in endothelial cell mitogenesis. DoD Breast Cancer Research Program Era of Hope Meeting. Atlanta, GA, June 8-12, p. 568.

Appendix 2

Yu, Y., Hulmes, J.D., Herley, M.T., Whitney, R.G., Crabb, J.W., and Sato, J.D. (2001) Direct identification of a major autophosphorylation site on vascular endothelial growth factor receptor Flt-1 that mediate phosphatidylinositol 3′-kinase binding. Biochem. J. In press.

Appendix 3

Approval letter from the Prince William Hospital IRB for human subjects protocol.

Appendix 4

Sato. J.D. (2000) Hybridoma cultures for production of antibodies. Purification and specificity of antibodies. In: Protocols for Neural Cell Cultures, (S. Fedoroff and A. Richardson, eds.), Human Press, New Jersey.

Appendix 5

Sato, J.D., Barnes, D.W., Hayashi, I., Hayashi, J., Hoshi, H., Kawamoto, T., McKeehan, W.L., Matsuda, R., Matsuzaki, K., Okamoto, T., Serrero, G., Sussman, D.J., and Kan, M. (2001) Specific cell types and their requirements. In: Basic Cell Culture: a Practical Approach, (J.M. Davis, ed.), Oxford University Press, Oxford. In press. (Title page only)

VEGF SIGNALING PATHWAYS IN ENDOTHELIAL CELL MITOGENESIS

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Vascular endothelial cell growth factor (VEGF)/vascular permeability factor (VPF) is a specific mitogen for endothelial cells. Since 1991 it has been known that VEGF, like many other polypeptide growth factors, activates a receptor-associated tyrosine kinase in target cells. We have used normal human umbilical vein endothelial cells (HUVECs) rather than immortal endothelial or non-endothelial cell lines transfected with VEGF receptor cDNAs to study intracellular signaling pathways activated by VEGF in endothelial cell mitogenesis. We assessed the activation of enzyme signaling intermediates by phosphorylation state and by increased kinase activity towards substrates. In addition, we used specific inhibitors of signaling enzymes to evaluate their significance in VEGF-stimulated cell proliferation. Like all polypeptide growth factors that have been tested VEGF rapidly and transiently activated the mitogen-activated protein kinases (MAPKs) ERKs 1 and 2. It also activated p38 MAP kinase in subconfluent HUVECs, but it had no detectable effect on stress-activated protein kinase (SAPK)/jun N-terminal kinase (JNK). Phosphatidylinositol 3-kinase (PI 3-kinase) and its downstream mediator p70 S6 kinase, phospholipase C gamma, and the delta and epsilon isoforms of protein kinase C were also activated by VEGF in HUVECs. By contrast, activation of the janus kinases (JAKs) and PKCs alpha and beta was not detected. The activities of ERKs 1 and 2, PI 3-kinase and p70 S6 kinase were specifically inhibited by PD98059, LY294002 and rapamycin, respectively, in VEGF-stimulated HUVECs, and each of the three inhibitors completely inhibited cell proliferation. There was no apparent cross-talk between PI 3-kinase or p70 S6 kinase and ERKs 1 and 2 or between the ERKs and p38 MAP kinase. Inhibition of p38 MAP kinase by the compound SB203580 resulted in the hyperphosphorylation of retinoblastoma (Rb) protein, and it stimulated HUVEC proliferation both in the presence and absence of VEGF. Our results indicate that the activities of the signaling enzymes ERKs 1 and 2, PI 3-kinase, and p70 S6 kinase are essential for VEGF-induced HUVEC proliferation while p38 MAP kinase acts to supress endothelial cell proliferation by inhibiting cell cycle progression. VEGF activates a multiplicity of signal transduction pathways in endothelial cells, but as none of these pathways is unique to VEGF, targeting VEGF receptors rather than intracellular signaling intermediates may be a more appropriate therapeutic strategy for controlling VEGF-induced cell proliferation in vivo.

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Direct identification of a major autophosphorylation site on vascular endothelial growth factor receptor Flt-1 that mediates phosphatidylinositol 3′-kinase binding

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Progress has been made in our understanding of the mechanism by which the binding of vascular endothelial growth factor (VEGF) to cognate receptors induces a range of biological responses, but it is far from complete. Identification of receptor autophosphorylation sites will allow us to determine how activated VEGF receptors are coupled to specific downstream signalling proteins. In the present study, we have expressed human VEGF receptors in insect cells using the baculovirus expression system, identified a major autophosphorylation site on the VEGF receptor fms-like tyrosine kinase-1 (Flt-1) by HPLC–electrospray ionization (ESI)–MS, and characterized *in vitro* interactions between Flt-1 and phosphatidylinositol 3′-kinase (PI3-kinase). Infection of High 5 insect cells with Flt-1 recombinant virus resulted in the expression of a 170 kDa glycoprotein, which bound VEGF with a K_d of 2×10^{-10} M in intact insect cells. The overexpressed recombinant Flt-1 receptors exhibited tyrosine kinase activity and were constitutively phosphorylated. Analysis of Flt-1 tryptic peptides by HPLC–ESI–MS with selective phosphate ion monitoring identified a hexapeptide (YVNAFK; where single-letter amino-acid code has been used) containing a phosphotyrosine (pTyr) residue at position 1213. Using synthetic phosphopeptides, this pTyr residue was found to be directly involved in the binding of PI3-kinase *in vitro* even though it did not fall within a consensus pYM/VXM PI3-kinase binding motif. These results suggest that phosphorylated Flt-1 associates with PI3-kinase at $pTyr^{1213}$ to mediate the activation of this pathway in VEGF signalling.

Key words: mass spectrometry, phosphotyrosine, receptor tyrosine kinase.

INTRODUCTION

The tyrosine kinase receptors fms-like tyrosine kinase-1 (Flt-1) and kinase insert domain-containing receptor [KDR; also known as fetal liver kinase-1 (Flk-1)], the two signal transmitting receptors for VEGF, play critical roles in angiogenesis. Gene targeting studies have shown that KDR/Flk-1 null mice lack blood islands and haematopoietic progenitor cells, while Flt-1 null mice have differentiated endothelial cells, but fail to form an organized embryonic vasculature [1,2]. These results indicated that Flt-1 and KDR/Flk-1 have distinct physiological roles during development and that both receptors are essential for survival.

Ligand binding activates receptor tyrosine kinases (RTKs) through receptor dimerization and autophosphorylation [3]. We demonstrated that VEGF activated a tyrosine kinase activity in human umbilical cord endothelial cells [4], and Flt-1 [5] and KDR [6] were subsequently characterized as VEGF-activated RTKs. Individual phosphotyrosine (pTyr) residues and adjacent amino acids generate highly selective binding sites for cytoplasmic signalling proteins containing Src homology (SH)2 or pTyr-binding ('PTB') domains [7]. Association of signalling proteins with phosphorylated receptors then results in the activation of intracellular signalling pathways, which induce gene expression and cellular responses [8]. Thus identification of VEGF receptor autophosphorylation sites will provide considerable insight into the interactions between VEGF receptors and downstream signalling proteins, and lead to a better understanding of the functions of individual VEGF receptors in regulating endothelial cells. We have recently reported that the mitogen-activated protein kinase (MAP kinase) and phosphatidylinositol 3′-kinase (PI3-kinase)/p70 S6 kinase pathways are essential for VEGF-induced endothelial cell proliferation while the p38 MAP kinase pathway regulates VEGF mitogenic activity by suppressing cell cycle progression [9]. However, the specific protein–protein interactions through which VEGF receptors activate specific downstream signalling pathways to transduce a mitogenic signal are not clear. Dougher-Vermazen et al. [10] found four pTyr residues ($pTyr^{951}$, $pTyr^{996}$, $pTyr^{1054}$ and $pTyr^{1059}$) in bacterially-expressed KDR kinase domain. Thus far, none of these pTyr residues has been specifically implicated in the binding of any signalling molecules, but KDR has been implicated in the activation of protein kinase B/Akt through PI3-kinase in endothelial cells [11], and experiments with the KDR inhibitor SU5416 suggest that KDR is involved in the activation of phospholipase C-γ (PLC-γ), protein kinase C-ε, and MAP kinase [12]. Several putative autophosphorylated tyrosine residues on Flt-1 were identified using either the yeast two-hybrid system or two-dimensional phospho amino acid analysis [13–17], but these reports made conflicting claims regarding phosphorylated tyrosine residues on Flt-1 and their potential binding proteins. Using the same technique, $pTyr^{1169}$ was identified in full-length Flt-1 [15], but not in overexpressed Flt-1 intracellular domain [17]. PLC-γ was found by Cunningham et al. [14], and Sawano et al.

Abbreviations used: ESI, electrospray ionization; FBS, fetal bovine serum; Flk-1, fetal liver kinase-1; Flt-1, fms-like tyrosine kinase-1; HUVECs, human umbilical vein endothelial cells; KDR, kinase insert domain-containing receptor; MAP kinase, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PI3-kinase, phosphatidylinositol 3′-kinase; PLC-γ, phospholipase C-γ; PNGase F, peptide N-glycosidase F; pTyr, phosphotyrosine; RTK, receptor tyrosine kinase; SH, Src homology; SIM, selective ion monitoring; VEGF, vascular endothelial cell growth factor.

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[15], to bind to pTyr[1100]; however, Ito et al. [17] reported that PLC-γ bound to pTyr[1213] and pTyr[1333]. Thus these experimental approaches have not yielded a consensus on either the identities of autophosphorylation sites or their interactions with signal transduction molecules. In the present study, we sought to identify autophosphorylated tyrosine residues on full-length recombinant human Flt-1 expressed in insect cells using a different technique, electrospray ionization (ESI)–MS, and to identify associated downstream signalling proteins.

ESI [18,19] was introduced as a novel way to volatilize peptides for mass analysis. When directly coupled to a chromatography system, on-line analysis of column eluates can be accomplished without the need for peptide purification. The use of triple quadrupole mass analysers allows the selection of parent ions of specific mass to charge ratio in the first quadrupole, which are then fragmented by collision-induced dissociation in the second quadrupole. A series of fragment ions that provide sequence information are measured in the third quadrupole. This process, known as tandem MS or MS–MS [20], can be applied to peptides in mixtures or in eluates from HPLC. Selective ion monitoring (SIM) monitors ion current at certain mass values throughout the complete mass spectrum as a function of time [21]. The combination of SIM with ESI–MS has been a powerful tool for identifying protein modifying groups at the post-translational level [22,23].

In the present study we report that Tyr[1213] is a major autophosphorylation site on Flt-1. Using synthetic phosphopeptides, we also show that PI3-kinase physically associates with Flt-1 at pTyr[1213]. These results indicate that Flt-1 participates in the regulation of endothelial cell functions through PI3-kinase-activated signalling pathways, and they are consistent with our previous finding that PI3-kinase signalling through p70 S6 kinase is required for VEGF-induced human endothelial cell proliferation [9].

EXPERIMENTAL

Reagents

Grace's insect cell culture medium, lactalbumin hydrolysate and yeastolate were purchased from Life Technologies (Gaithersburg, MD, U.S.A.). Fetal bovine serum (FBS) was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Sf9 and High 5 insect cells, and the pVL1392 baculovirus transfer vector were obtained from Invitrogen (Carlsbad, CA, U.S.A.). Anti-(Flt-1) (C-17) and anti-(Flk-1) (C-1158) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal anti-pTyr antibody (4G10) and monoclonal anti-(PI3-kinase) (p85α) were purchased from Upstate Biotechnology. Protein A–Sepharose, Protein G–agarose and sulfolink coupling gel were from Pierce Chemical Co. (Rockford, IL, U.S.A.). PVDF membrane was from Millipore (Bedford, MA, U.S.A.), and ECL® Western blotting detection reagents were obtained from Amersham (Little Chalfont, Bucks., U.K.). [γ-^{32}P]ATP and ^{35}S-protein labelling mix were from NEN Life Science Products (Boston, MA, U.S.A.). Na_3VO_4 and the protease inhibitors leupeptin, pepstatin A, aprotinin and PMSF were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetonitrile and trifluoroacetic acid were obtained from Fisher Scientific (Springfield, NJ, U.S.A.).

Cell culture

Sf9 and High 5 insect cells were maintained at 27 °C in Grace's insect culture medium supplemented with lactalbumin hydrolysate, yeastolate and 10% (v/v) FBS. Human umbilical vein endothelial cells (HUVECs) were isolated and maintained as previously described [9].

RNA isolation and reverse transcription

Total RNA was prepared from HUVECs using RNAzol (Biotecx Laboratories, Houston, TX, U.S.A.). cDNA templates for PCR were synthesized from 5 μg of total RNA with oligo(dT) primers using a SuperScript preamplification system (Life Technologies).

Cloning and sequencing of VEGF receptor cDNAs

cDNA fragments (1.6 kb) encoding the intracellular portion of Flt-1 or KDR were cloned using PCR. The following synthetic oligonucleotide primer pairs were used for Flt-1 (forward, 5′-CCTTATGATGCCAGCAAGTGG-3′; reverse, 5′-CTGGATCCGGCTTCGTGTCAAACTCTAG-3′) and KDR (forward, 5′-CATGGATCCAGATGAACTCCCATTGG-3′; reverse, 5′-CTGGATCCTTAAACAGGAGGAGAGCTC-3′). Full-length receptor cDNAs were made by splicing the 1.6 kb Flt-1 fragment on to a 2.4 kb cDNA fragment encoding the extracellular portion of Flt-1 in the pVL1392 transfer vector [24] cut with the restriction enzymes *Pfl*MI and *Bam*HI, and by splicing the 1.6 kb KDR fragment on to a 2.4 kb cDNA fragment encoding the extracellular portion of KDR in the pVL1392 vector [24] at a unique *Bam*HI site. Samples for sequence analysis were prepared using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (PE Biosystems, Foster City, CA, U.S.A.). The complete cDNAs of KDR and Flt-1 were sequenced on an Applied Biosystems Model 370A DNA sequencer.

Antibodies

Rabbit polyclonal antisera P20L and K20L were raised against the synthetic peptides PNNQSGSEQRVEVTECSDGL and KDPELSLKGTQHIMQAGQTL (where single-letter amino-acid code has been used) corresponding to N-terminal amino acid sequences of human KDR and Flt-1 respectively. The peptides were synthesized by the Adirondack Biomedial Research Institute Protein Chemistry Facility. Immunization of rabbits with keyhole-limpet haemocyanin-conjugated peptides and collection of sera were performed by Cocalico Biologicals (Reamstown, PA, U.S.A.). The antibodies were affinity-purified using peptide-coupled Affi-gel 10 (Bio-Rad).

Generation of recombinant baculovirus and infection of High 5 cells

Recombinant viruses were generated by cotransfection of Sf9 cells with PVL1392/Flt-1 or PVL1392/KDR and Baculogold baculovirus DNA (BD Pharmingen, San Diego, CA, U.S.A.) using lipofectin (Life Technologies). Recombinant viruses were purified by plaque assay [25], and were verified by PCR. Cells were lysed and the recombinant proteins were analysed by immunoprecipitation and/or immunoblotting 48 h post-infection.

Metabolic labelling of recombinant Flt-1 and KDR

Subconfluent High 5 cells in a 24 well plate (Corning) were infected with recombinant viruses. Prior to labelling, the cells were incubated with methionine-deficient medium (Life Technologies) for 1 h. The same medium containing 25 μCi of ^{35}S-protein labelling mix (NEN Life Science Products) was added, 2 h before each time point. Incubation was continued at 27 °C

leupeptin, 10 μg/ml aprotinin and 1 μg/ml pepstatin. Cell lysates were clarified by centrifugation twice at 13000 g for 15 min. A 50% (v/v) peptide–Sulfolink gel slurry (30 μl) containing 3 μg of peptide was incubated with 0.5 mg of HUVEC lysate protein at 4 °C for 2 h. The gel beads were washed with lysis buffer containing 0.2 M NaCl, and the bound proteins were analysed by Western blotting with a monoclonal antibody directed against PI3-kinase.

RESULTS

Molecular cloning of Flt-1 cDNA

The full-length coding region cDNA for Flt-1 was amplified from HUVEC RNA by reverse transcription PCR using Taq and Vent polymerases, and it was subcloned into the baculovirus transfer vector PVL1392. Two nucleotide differences were found between our Flt-1 sequence and the original published sequence [28], resulting in a single amino acid difference (Leu^{770} → Phe) that was also reported by de Vries et al. [5]. For comparison KDR coding region cDNA was also cloned and sequenced. The Flt-1 and KDR cDNA sequences have been deposited with GenBank® (accession numbers AF063657 and AF063658).

Recombinant Flt-1 as a glycosylated membrane-bound protein with specific binding affinity for VEGF

Recombinant Flt-1 expressed in insect cells was detected in cell lysates by Western blotting as a single protein of approx. 170 kDa, whereas recombinant KDR was detected as a doublet of approx. 190 and 170 kDa (Figure 1). To determine whether recombinant Flt-1 was post-translationally glycosylated, similarly to endogenous Flt-1 in endothelial cells, membrane proteins prepared from infected High 5 cells were digested with peptide N-glycosidase F (PNGase F). Recombinant Flt-1 was essentially completely digested by this enzyme, reducing the apparent molecular mass estimated by SDS/PAGE from 170 to 155 kDa, which was close to the 150 kDa mass predicted for the non-glycosylated Flt-1 polypeptide (Figure 2). Recombinant KDR was similarly deglycosylated by PNGase F. It is noteworthy that in cell membranes, KDR was present as a single 190 kDa protein, which corresponded to the higher molecular mass KDR polypeptide detected in whole-cell lysates (Figure 1). When KDR-infected High 5 cells were incubated in the presense of the glycosylation inhibitor tunicamycin, only the lower molecular mass KDR was expressed (results not shown) indicating that the 170 kDa KDR was not glycosylated. Recombinant Flt-1 bound to the 165-amino-acid isoform of VEGF in a saturable manner (Figure 3).

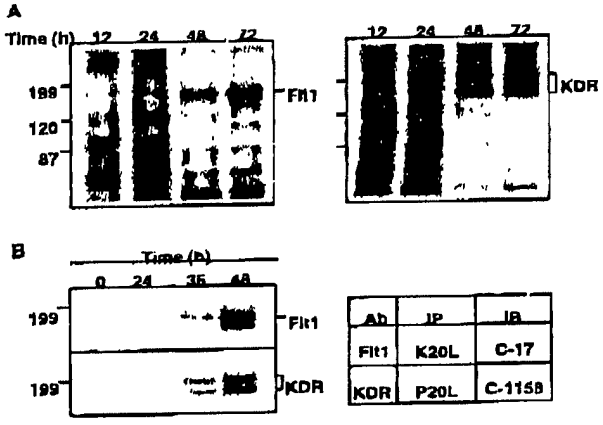

Figure 1 Time course of Flt-1 and KDR expression in baculovirus-infected High 5 cells

(A) Autoradiogram of ^{35}S-labelled proteins of cells that were pulse-labelled for 2 h and lysed at the indicated time points. Detergent lysates were resolved by SDS/PAGE, and the dried gel was exposed to X-ray film. (B) Flt-1 and KDR were immunoprecipitated (IP) from detergent lysates at 0, 24, 36 and 48 h post-infection, and were subjected to SDS/PAGE. They were then detected by immunoblotting (IB). The antibodies (Ab) used are listed. Molecular masses of protein standards are indicated on the left-hand sides of the panels.

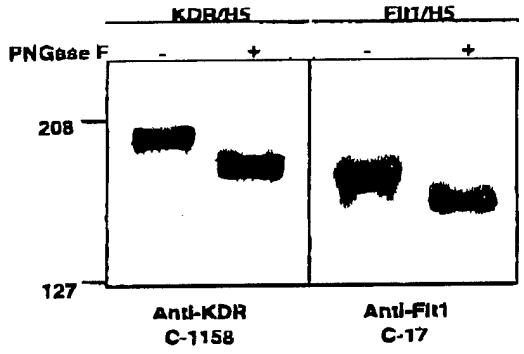

Figure 2 PNGase F digestion of Flt-1 and KDR expressed in High 5 insect cells

Recombinant virus-infected High 5 (H5) cells were lysed 48 h post-infection. Cells were fractionated by homogenization, and membranes were collected by untracentrifugation. Digestion was carred out by incubating membrane proteins with (+) or without (−) PNGase F at 37 °C overnight. Flt-1 or KDR in the digestion mixture was analysed by Western blotting with the indicated anti-peptide antibodies. Molecular masses of protein standards are indicated on the left-hand side.

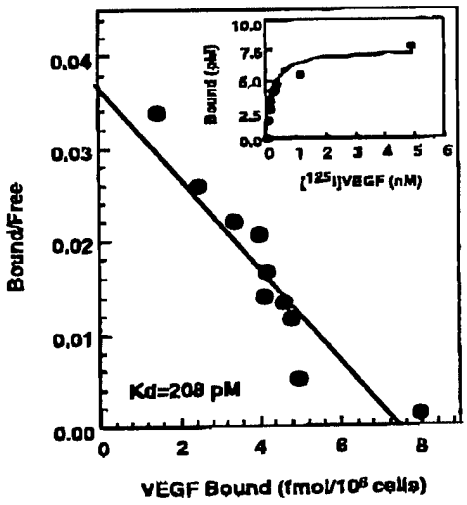

Figure 3 Analysis of VEGF binding to intact High 5 cells expressing Flt-1

Scatchard plot of the saturation binding assay (shown in the inset). Increasing amounts of [^{125}I]VEGF were added to Flt-1 virus-infected High 5 cells that had been fixed in 48 well plates. Non-specific binding was determined by competition with a 100-fold excess of unlabelled VEGF. After binding, the cells were washed and lysed, and the cell-associated radioactivity was determined using a gamma counter. Results are presented as the means of triplicate determinations.

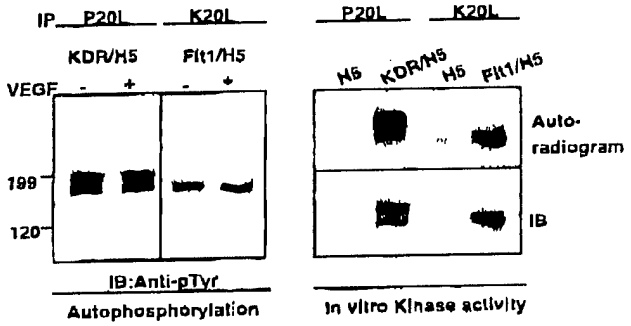

Figure 4 Ligand-independent VEGF receptor tyrosine phosphorylation in Flt-1- or KDR-infected High 5 (H5) cells

Left-hand panel: at 48 h post-infection, cells were treated with (+) or without (—) 50 ng/ml VEGF for 5 min. Flt-1 or KDR was immunoprecipitated (IP) from whole-cell lysates with anti-peptide antibodies K20L or P20L, and analysed by Western blotting with anti-pTyr antibodies. Molecular masses of protein standards are indicated on the left-hand side. Right-hand panel: Flt-1 or KDR immunoprecipitates were subjected to an *in vitro* kinase assay. Samples were analysed by SDS/PAGE, and transferred on to a PVDF membrane, followed by KOH treatment and autoradiography. Flt-1 or KDR was detected on the membrane by immunoblotting (IB) with anti-(Flt-1) (C-17) or anti-KDR (C-1158) antibodies.

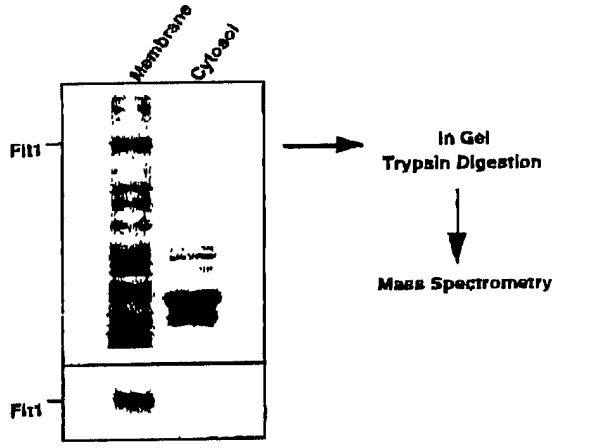

Figure 5 Preparation of baculovirus-expressed recombinant Flt-1 for analysis by MS

Flt-1-infected High 5 cells (48 h post-infection) were fractionated by homogenization and ultracentrifugation. Membrane and cytosolic proteins were resolved by SDS/PAGE [7.5% (w/v) polyacrylamide]. Flt-1 was located in the gel stained with Commassie Brilliant Blue (top panel). A parallel gel (bottom panel) was transferred on to a PVDF membrane and immunoblotted with anti-(Flt-1) (C-17) antibodies. The immunoreactive Flt-1 corresponds to a 170 kDa band in the stained gel. Flt-1 protein bands were excised from the stained gel, digested with trypsin and analysed using an API 300 HPLC–tandem MS mass spectrometer.

Scatchard analysis revealed the presence of a single class of high-affinity binding sites on these cells with a dissociation constant (K_d) of 2×10^{-10} M, indicating that the recombinant Flt-1 was intact and functional.

Ligand-independent tyrosine phosphorylation of recombinant Flt-1

Western blotting with an anti-pTyr antibody of anti-receptor immunoprecipitates of infected cells treated with or without VEGF indicated that both Flt-1 and KDR were tyrosine phosphorylated in the absence of VEGF (Figure 4, left-hand panel). These phosphorylation events resulted from receptor autophosphorylation and not from the activity of unrelated tyrosine kinases as demonstrated recently by Sawano et al. [15] with an Flt-1 kinase-negative mutant, which exhibited no tyrosine phosphorylation in insect cells. Ligand-independent autophosphorylation of RTKs has been previously reported for platelet-derived growth factor (PDGF) receptors [29,30], epidermal growth factor receptors [31,32] and fibroblast growth factor receptors [33] expressed in the baculovirus system. The intrinsic kinase activity of recombinant Flt-1 or KDR was confirmed in an *in vitro* kinase assay (Figure 4, right-hand panel). These results indicated that the baculovirus system was a useful model with which to study the autophosphorylation sites of the Flt-1 receptor, whose tyrosine kinase activity is difficult to detect in a mammalian cell environment [34,35].

Identification of autophosphorylation sites on Flt-1

To produce sufficient amounts of tyrosine autophosphorylated recombinant Flt-1, we initially tried to purify Flt-1 from insect cells by affinity chromatography on a VEGF column. Flt-1 receptors were first enriched by isolating plasma membranes from infected High 5 cells pretreated with VEGF. In tests with a variety of detergents, the efficiency of solubilizing Flt-1 from the membranes, however, was extremely low. We then took an alternative approach and purified Flt-1 by SDS/PAGE. Membrane-derived 170 kDa Flt-1 protein was clearly visualized by Commassie Brilliant Blue on an SDS/polyacrylamide gel (Figure 5, top panel). In a parallel gel, this 170 kDa protein was recognized by anti-(Flt-1) antibodies (Figure 5, bottom panel) and by an anti-pTyr antibody (results not shown). After trypsin digestion of Flt-1 protein bands excised from the stained gel, tryptic peptide extracts were run on HPLC–MS.

The tryptic digests were analysed by capillary liquid chromatography coupled with ESI–MS. Flt-1 peptides were identified in HPLC–MS chromatograms by comparing observed masses with those of predicted proteolytic fragments. Peptide sequences were confirmed by tandem MS. Phosphorylated peptides were identified by SIM, in which phosphate groups were detected in the first quadrupole of the mass spectrometer as a result of partial dephosphorylation. This was achieved by applying a high orifice potential to a portion of each sample. pTyr-containing peptides were then detected where phosphate ions (PO_3^- in negative ion mode) were identified by mass increases of 80 Da. The only phosphopeptide detected was the tryptic hexapeptide YVNAFK (Figure 6), which contained a phosphorylated tyrosine residue ($pTyr^{1213}$) derived from a region near the C-terminus of Flt-1.

Flt-1 interaction with PI3-kinase is determined by Tyr^{1213}

The three amino acids immediately C-terminal to an RTK pTyr residue define the binding specificity for SH2 domain-containing proteins [8,36]. The phosphopeptide pYVNA of Flt-1 fits the general binding motif pTyr-hydrophobic-Xaa-hydrophobic elucidated for PI3-kinase, PLC-γ and SH protein-tyrosine phosphatase 2 ('SHPTP2') [36]. Cunningham et al. [13] inferred that Flt-1 bound PI3-kinase through $pTyr^{1213}$ from results obtained with Flt-1 kinase domain and PI3-kinase SH2 domain constructs in the yeast two-hybrid system. In addition, we have reported that VEGF activated the PI3-kinase pathway in human endothelial cell mitogenesis [9]. From these results, PI3-kinase appeared to be a potential signalling protein that could be associated with autophosphorylated Flt-1.

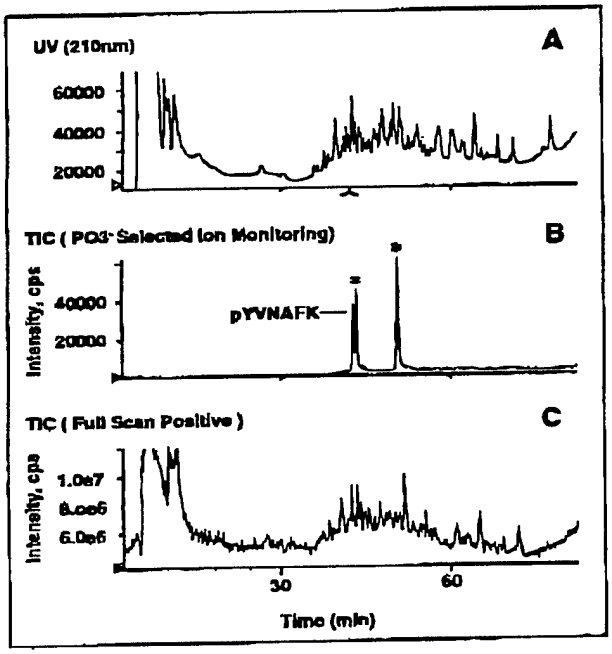

Figure 6 HPLC–ESI–MS analysis of tryptic Flt-1 VEGF receptor

Approx. 10 pmol of an Flt-1 tryptic digest were analysed by HPLC–ESI–MS with SIM for phosphopeptides (PO_3^- in negative ion mode). (**A–C**) Are from one run using a 140D capillary HPLC system (Applied Biosystems) and a 0.5 mm × 150 mm C_{18} column (Applied Biosystems) at a flow rate of 5 μl/min. *Biotin-ApSSYK (10 pmol) and EPQpYEEIPIA-NH_2 (4 pmol) were injected with the digestion mixture as internal positive control phosphopeptides. All of the column effluent was analysed with an API 300 ESI mass spectrometer. (**A**) UV absorbance profile at 210 nm. (**B**) Negative ion detection profile for PO_3^-. (**C**) Full scan positive total ion current (TIC). The pTyr-containing hexapeptide pYVNAFK is indicated. 6.0e6 = 6.0×10^6.

To demonstrate a direct physical interaction between Flt-1 and PI3-kinase, with the involvement of $pTyr^{1213}$, *in vitro* binding studies were performed. Two 15-residue peptides corresponding to Flt-1 amino acid residues 1206–1219 were synthesized, in which Tyr^{1213} was phosphorylated (CG15FpY) or left unmodified (CG15F) (see Figure 7A). To determine whether autophosphorylated recombinant Flt-1 associated with mammalian PI3-kinase, Flt-1 immunoprecipitates from infected High 5 cells were extensively washed and incubated with lysates from non-stimulated HUVECs in the presence of CG15F or CG15FpY peptides (Figure 7B, right-hand panel). Western-blot analysis of bound proteins with a specific antibody raised against PI3-kinase revealed that recombinant full-length Flt-1 associated with PI3-kinase, and the binding was competely blocked by the phosphopeptide CG15FpY, but not by the control peptide CG15F. Unrelated pTyr-containing peptides of similar length also had no effect on the co-immunoprecipitation of Flt-1 and PI3-kinase (results not shown). These results indicated that the interaction with PI3-kinase was dependent on the phosphorylation of Tyr^{1213}. The requirement of $pTyr^{1213}$ for the binding of PI3-kinase was also investigated using immobilized phosphopeptides. CG15F and CG15FpY were covalently coupled to Sulfolink agarose gel through their free N-terminal cysteine residues, and they were incubated with HUVEC lysates. The peptide-coupled Sulfolink gel was then collected by centrifugation and washed, and bound proteins were analysed by Western blotting with an anti-(PI3-kinase) antibody. As shown in Figure 7(B) (left-hand panel), the

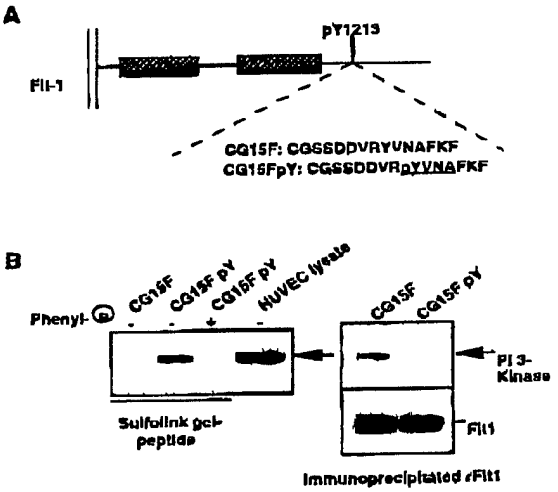

Figure 7 *In vitro* association of PI3-kinase with Flt-1 receptors at $pTyr^{1213}$

(**A**) The location of Tyr^{1213} in Flt-1 and the sequences of synthetic peptides CG15F and CG15FpY. The two hatched boxes represent the split tyrosine kinase domain of the Flt-1 receptor. (**B**) Right-hand panel: Recombinant Flt-1 receptors were immunoprecipitated from infected High 5 cells at 48 h post-infection with specific antibodies (K20L) to Flt-1 and incubated with cell lysates of non-stimulated HUVECs in the presence of peptide CG15F or CG15FpY. Bound proteins were analysed by Western blotting with a monoclonal anti-(PI3-kinase) antibody. The position of PI3-kinase is marked with an arrow. Left-hand panel: HUVEC lysates were incubated with the peptides coupled to Sulfolink gel in the absence (−) or presence (+) of 40 mM phenylphosphate. Bound proteins were analysed by Western blotting.

CG15FpY-coupled gel bound PI3-kinase, whereas the non-phosphorylated control peptide exhibited no detectable binding to PI3-kinase. Furthermore, the association of the immobilized phosphopeptide with the PI3-kinase was abolished when phenylphosphate was added to the incubation, indicating that the protein–protein interaction was mediated via $pTyr^{1213}$. Western blotting also showed that the CG15pY phosphopeptide bound PLC-γ (results not shown), which is in agreement with the results of Ito et al. [17]. Our data demonstrate that PI3-kinase binds directly to Flt-1, and this interaction is mediated specifically by $pTyr^{1213}$.

DISCUSSION

A role for PI3-kinase in VEGF-induced cell signalling has been controversial. Although PI3-kinase is activated by a number of polypeptide growth factors [37], previous investigations found that PI3-kinase was not activated by VEGF in VEGF receptor cDNA-transfected porcine endothelial cells [34], Flt-1 cDNA-transfected NIH 3T3 fibroblasts [35], HUVECs [38], sinusoidal endothelial cells [39], or mouse capillary endothelial cells [17]. In support of a role for PI3-kinase in VEGF signalling, the p85 subunit of PI3-kinase was phosphorylated in bovine endothelial cells in response to VEGF [40], and it bound to $pTyr^{1213}$ in the intracellular domain of Flt-1 in a yeast two-hybrid assay [13]. Subsequently, VEGF-stimulated PI3-kinase activity was shown to be important in the anti-apoptotic [11] and the proliferative [9,41] responses of human endothelial cells to VEGF. The present study provides additional evidence that PI3-kinase could mediate VEGF signalling through a direct physical interaction with activated Flt-1.

We have used HPLC–ESI–MS to identify Tyr^{1213} as the major autophosphorylation site on Flt-1. Previously, two different approaches were taken to identify Flt-1 phosphorylation sites. Using the yeast two-hybrid method coupled with site-directed mutagenesis, Cunningham et al. [13,14] identified Tyr^{1213}, Tyr^{1169} and Tyr^{794} as residues modified by phosphorylation, whereas Igarashi et al. [16] identified Tyr^{1213} and Tyr^{1333} as phosphorylation sites. Phospho amino acid analysis of wild-type and mutant tryptic peptides separated by two-dimensional gel electrophoresis indicated that Tyr^{1213} and Tyr^{1169} were autophosphorylated on full-length Flt-1 expressed in insect cells [15]. However, using the same method Ito et al. [17] reported four phosphorylation sites, Tyr^{1213}, Tyr^{1242}, Tyr^{1327} and Tyr^{1333}, but not Tyr^{1169}, on the baculovirus expressed Flt-1 kinase domain. Possible explanations for these discrepancies are differences in experimental conditions, assay sensitivities and receptor regions expressed (i.e. full-length receptor or tyrosine kinase domain). In each of these reports Flt-1 phosphorylation sites were identified by indirect methods. In the present study we have used MS to identify autophosphorylated Tyr^{1213} on recombinant full-length Flt-1 protein. No other phosphorylated tryptic peptides were detected. We cannot exclude the possibility that the phosphorylation levels of other Flt-1 tyrosine residues expressed in High 5 cells were below the detection limit of the MS system used. Although Tyr^{1213} was previously identified as a putative autophosphorylation site of Flt-1 that binds PI3-kinase [13,16], no direct evidence for this interaction was provided. In the present study we demonstrated an interaction between PI3-kinase and Tyr^{1213} with *in vitro* binding experiments using a synthetic phosphopeptide containing $pTyr^{1213}$. The same phosphopeptide sequence also bound PLC-γ as shown by Ito et al. [17] (results not shown). PI3-kinase is composed of a p85 regulatory subunit, which contains two SH2 domains and one SH3 domain, and the p110 catalytic domain [42]. Since VEGF stimulates the phosphorylation of the p85 subunit of PI3-kinase in HUVECs [41] and induces kinase activity [9,43], the binding of inactive PI3-kinase to Flt-1 is most likely mediated by the SH2 domains of the p85 subunit as suggested by Cunningham et al. [13]. The YVNA sequence of Flt-1 that includes Tyr^{1213} is different from the YM/VXM motif for PI3-kinase that has been identified in other related RTKs, including the PDGF receptor, the colony-stimulating factor-1 receptor and c-kit. Thus there is more variability in the RTK binding motif recognized by PI3-kinase than previously appreciated.

We have previously shown that VEGF-activated PI3-kinase in HUVECs phosphorylates phosphotidylinositol, and this kinase plays an essential role in transducing a VEGF mitogenic signal [9]. The results reported in the present study suggest that the binding of activated Flt-1 through $pTyr^{1213}$ to PI3-kinase contributes to the activation of PI3-kinase. This protein–protein interaction therefore couples an extracellular VEGF stimulus to the intracellular PI3-kinase signal transduction pathway in vascular endothelial cells.

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REFERENCES

1 Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L. and Schuh, A. C. (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature (London) **376**, 62–66

2 Fong, G. H., Rossant, J., Gertsenstein, M. and Breitman, M. L. (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature (London) **376**, 66–70

3 Heldin, C.-H. (1995) Dimerization of cell surface receptors in signal transduction. Cell (Cambridge, Mass.) **80**, 213–223

4 Myoken, Y., Kayada, Y., Okamoto, T., Kan, M., Sato, G. H. and Sato, J. D. (1991) Vascular endothelial cell growth factor (VEGF) produced by A-431 human epidermoid carcinoma cells and identification of VEGF membrane binding sites. Proc. Natl. Acad. Sci. U.S.A. **88**, 5819–5823

5 de Vries, C., Escobedo, J. A., Houck, K., Ferrara, N. and Williams, L. T. (1992) The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science (Washington, D.C.) **255**, 989–991

6 Terman, B. I., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D. and Bohlen, P. (1992) Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem. Biophys. Res. Commun. **187**, 1579–1586

7 Kavanaugh, W. M., Turck, C. W. and Williams, L. T. (1995) PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine. Science (Washington, D.C.) **268**, 1177–1179

8 Fantl, W. J., Johnson, D. E. and Williams, L. T. (1993) Signaling by receptor tyrosine kinases. Annu. Rev. Biochem. **62**, 453–481

9 Yu, Y. and Sato, J. D. (1999) MAP kinases, phosphatidylinositol 3-kinase, and p70 S6 kinase mediate the mitogenic response of human endothelial cells to vascular endothelial growth factor. J. Cell. Physiol. **178**, 235–246

10 Dougher-Vermazen, M., Hulmes, J. D., Bohlen, P. and Terman, B. I. (1994) Biological activity and phosphorylation sites of the bacterially expressed cytosolic domain of the KDR VEGF-receptor. Biochem. Biophys. Res. Commun. **205**, 728–738

11 Gerber, H-P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B. A., Dixit, V. and Ferrara, N. (1998) Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3-kinase/Akt signal transduction pathway. J. Biol. Chem. **273**, 30336–30343

12 Wu, L.-W., Mayo, L. D., Dunbar, J. D., Kessler, K. M., Baerwald, M. R., Jaffe, E. A., Wang, D., Warren, R. S. and Donner, D. B. (2000) Utilization of distinct signaling pathways by receptors for vascular endothelial cell growth factor and other mitogens in the induction of endothelial cell proliferation. J. Biol. Chem. **275**, 5096–5103

13 Cunningham, S. A., Waxham, M. N., Arrate, P. M. and Brock, T. A. (1995) Interaction of the Flt-1 tyrosine kinase receptor with the p85 subunit of phosphatidylinositol 3-kinase. Mapping of a novel site involved in binding. J. Biol. Chem. **270**, 20254–20257

14 Cunningham, S. A., Arrate, M. P., Brock, T. A. and Waxham, M. N. (1997) Interactions of FLT-1 and KDR with phospholipase C-γ: identification of the phosphotyrosine binding sites. Biochem. Biophys. Res. Commun. **240**, 635–639

15 Sawano, A., Takahashi, T., Yamaguchi, S. and Shibuya, M. (1997) The phosphorylated 1169-tyrosine containing region of Flt-1 kinase (VEGFR-1) is a major binding site for PLC-γ. Biochem. Biophys. Res. Commun. **238**, 487–491

16 Igarashi, K., Isohara, T., Kato, T., Shigeta, K., Yamano, T. and Uno, I. (1998) Tyrosine 1213 of Flt-1 is a major binding site of Nck and SHP-2. Biochem. Biophys. Res. Commun. **245**, 95–99

17 Ito, N., Wernstedt, C., Engstrom, U. and Claesson-Welsh, L. (1998) Identification of vascular endothelial growth factor receptor-1 tyrosine phosphorylation sites and binding of SH2 domain-containing molecules. J. Biol. Chem. **273**, 23410–23418

18 Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F. and Whitehouse, C. M. (1989) Electrospray ionization for mass spectrometry of large biomolecules. Science (Washington, D.C.) **246**, 64–71

19 Edmonds, C. G. and Smith, R. D. (1990) Electrospray ionization mass spectrometry. Methods Enzymol. **193**, 412–431

20 Yost, R. A. and Boyd, R. K. (1990) Tandem mass spectrometry: quadrupole and hybrid instruments. Methods Enzymol. **193**, 154–200

21 Watson, J. T. (1990) Selected-ion measurements. Methods Enzymol. **193**, 86–106

22 Ding, J., Burkhart, W. and Kassel, D. B. (1994) Identification of phosphorylated peptides from complex mixtures using negative-ion orifice-potential stepping and capillary liquid chromatography/electrospray ionization mass spectrometry. Rapid Commun. Mass Spectrom. **8**, 94–98

23 Townsend, R. R., Lipniunas, P. H., Tulk, B. M. and Verkman, A. S. (1996) Identification of protein kinase A phosphorylation sites on NBD1 and R domains of CFTR using electrospray mass spectrometry with selective phosphate ion monitoring. Protein Sci. **5**, 1865–1873

24 Herley, M. T., Yu, Y., Whitney, R. G. and Sato, J. D. (1999) Characterization of the VEGF binding site on the Flt-1 receptor. Biochem. Biophys. Res. Commun. **262**, 731–738

25 O'Reilly, D. R., Miller, L. K. and Luckow, V. A. (1992) Baculovirus Expression Vector: A Laboratory Manual, CRC Press, Boca Raton, Florida

26 Kamps, M. P. and Sefton, B. M. (1989) Acid and base hydrolysis of phosphoproteins bound to Immobilon facilitates analysis of phosphoamino acids in gel-fractionated proteins. Anal. Biochem. **176**, 22–27

27 Chapline, C., Cottom, J., Tobin, H., Hulmes, J., Crabb, J. and Jaken, S. (1998) A major, transformation-sensitive PKC-binding protein is also a PKC substrate involved in cytoskeletal remodeling. J. Biol. Chem. **273**, 19482–19489

28 Shibuya, M., Yamaguchi, S., Yamane, A., Ikeda, T., Tojo, A., Matsushime, H. and Sato, M. (1990) Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene **5**, 519–524
29 Jensen, R. A., Beeler, J. F., Heidaran, M. A. and LaRochelle, W. J. (1992) Characterization of baculovirus-expressed human α and β platelet-derived growth factor receptors. Biochemistry **31**, 10887–10892
30 Roche, S., Dhand, R., Waterfield, M. D. and Courtneidge, S. A. (1994) The catalytic subunit of phosphatidylinositol 3-kinase is a substrate for the activated platelet-derived growth factor receptor, but not for middle-T antigen-pp60$^{c\text{-}src}$ complexes. Biochem. J. **301**, 703–711
31 Greenfield, C., Patel, G., Clark, S., Jones, N. and Waterfield, M. D. (1988) Expression of the human EGF receptor with ligand-stimulatable kinase activity in insect cells using a baculovirus vector. EMBO J. **7**, 139–146
32 Meisenhelder, J. and Hunter, T. (1992) The SH2/SH3 domain-containing protein Nck is recognized by certain anti-phospholipase C-γ 1 monoclonal antibodies, and its phosphorylation on tyrosine is stimulated by platelet-derived growth factor and epidermal growth factor treatment. Mol. Cell. Biol. **12**, 5843–5856
33 Shi, E., Kan, M., Xu, J., Wang, F., Hou, J. and McKeehan, W. L. (1993) Control of fibroblast growth factor receptor kinase signal transduction by heterodimerization of combinatorial splice variants. Mol. Cell. Biol. **13**, 3907–3918
34 Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M. and Heldin, C.-H. (1994) Different signal transduction properties of KDR and Flt-1, two receptors for vascular endothelial growth factor. J. Biol. Chem. **269**, 26988–26995
35 Seetharam, L., Gotoh, N., Maru, Y., Neufeld, G., Yamaguchi, S. and Shibuya, M. (1995) A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. Oncogene **10**, 135–147
36 Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J. et al. (1993) SH2 domains recognize specific phosphopeptide sequences. Cell (Cambridge, Mass.) **72**, 767–778
37 Roche, S., Koegl, M. and Courtneidge, S. A. (1994) The phosphatidylinositol 3-kinase α is required for DNA synthesis induced by some, but not all, growth factors. Proc. Natl. Acad. Sci. U.S.A. **91**, 9185–9189
38 Abedi, H. and Zachary, I. (1997) Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. J. Biol. Chem. **272**, 15442–15451
39 Takahashi, T. and Shibuya, M. (1997) The 230 kDa mature form of KDR/Flk-1 (VEGF receptor-2) activates the PLC-γ pathway and partially induces mitotic signals in NIH3T3 fibroblasts. Oncogene **14**, 2079–2089
40 Guo, D., Jia, Q., Song, H.-Y., Warren, R. S. and Donner, D. B. (1995) Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains. Association with endothelial cell proliferation. J. Biol. Chem. **270**, 6729–6733
41 Thakker, G. D., Hajjar, D. P., Muller, W. A. and Rosengart, T. K. (1999) The role of phosphatidylinositol 3-kinase in vascular endothelial growth factor signaling. J. Biol. Chem. **274**, 10002–10007
42 Marengere, L. E. M. and Pawson, T. (1994) Structure and function of SH2 domains. J. Cell Sci. Suppl. **18**, 97–104
43 Gille, H., Kowalski, J., Li, B., LeCouter, J., Moffat, B., Zioncheck, T. F., Pelletier, N. and Ferrara, N. (2001) Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. J. Biol. Chem. **276**, 3222–3230

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PRINCE WILLIAM HEALTH SYSTEM

June 14, 2001

J. Denry Sato, D. Phil.
Research Scientist
American Type Culture Collection (ATCC)
10801 University Boulevard
Manassas, VA 20110

Dear Dr. Sato:

On May 18, 2001 the Prince William Hospital Human Research Review Committee approved your study entitled "Receptor Monoclonoal Antibodies that Inhibit Tumor Angiogenesis".

I am enclosing a copy of the updated Consent Form and have included a list of the changes that were made to the document. If these changes meet with your approval, we should be ready to begin the study. However, if any further changes are needed, this will delay the start date of the study.

If you are comfortable with the changes and are ready to begin the study, please contact Virginia Blair's office at 369-8270.

We look forward to getting this study up and running and working with ATCC on this project.

Sincerely,

James Fletcher/tmb

James Fletcher, Ph.D.
Chairman, HRRC

Enclosures

JF/tmb

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Chapter Twenty-Four

Hybridoma Cultures for Production of Antibodies

Purification and Specificity of Antibodies

J. Denry Sato

1. INTRODUCTION

During the process of characterizing the structure and function of novel antigens, it is usually necessary to create new monoclonal or polyclonal antibody reagents. However, once validated, these antibodies can be put to a multitude of experimental uses, such as detecting and quantitating antigens in cell and tissue extracts or biological fluids, purifying proteins for structural analyses, studying protein–protein interactions, and monitoring cellular differentiation. Although many well-characterized monoclonal antibodies (mAbs) to antigens of general interest are commercially available, mAbs to specialty antigens may need to be individually purified from hybridomas obtained from academic sources, or from not-for-profit cell repositories, such as the American Type Culture Collection (www.atcc.org). This chapter describes protocols for producing, purifying, and verifying murine mAbs from pre-existing hybridomas.

2. PRODUCTION OF MABS

mAbs can be collected from hybridomas cultured either in vitro or in the peritoneal cavities of major histocompatibility complex-compatible or immunodeficient strains of mice. Although mAbs can be recovered at high concentrations in ascites fluid, those antibodies are contaminated with an unknown proportion of irrelevant antibodies from the host mice. Because there are applications in which accurate estimates of antibody concentrations are important, we prefer to collect mAbs in serum-free cell culture medium. The medium formulation provided below is a modification of a serum-free medium developed for NS-1-Ag4-1 and related mouse myeloma cell lines (Kawamoto et al., 1986; Sato et al., 1987; Myoken et al., 1989).

2.1. Materials

RPMI-1640 DMEM nutrient medium plus five factors (RD+5F).
50X BSA-oleic acid stock solution.

Protocols for Neural Cell Culture, 3rd Ed. • Ed.: S. Fedoroff and A. Richardson • Humana Press, Inc., Totowa, NJ

Roller bottles, 2-L.
Pipets, disposable, plastic.
Tubes, conical, polypropylene, sterile, 50-mL.
95% air/5% CO_2, sterile.

2.2. Procedure

1. Add 250 mL RD+5F medium to each 2-L roller bottle. Add BSA-oleic acid stock solution (1X-1 mg/mL BSA and 10 µg/mL oleic acid).
2. Collect hybridoma cells in 50-mL conical tubes, and centrifuge for 5 min at 200*g*. Add the cells to the roller bottles at a final density of 1–5 + 10^5 cells/mL medium.
3. Gas the medium with sterile 95% air/5% CO_2 to prevent the pH from increasing, and tighten the roller bottle caps, to prevent gas exchange.
4. Incubate the roller bottles at 37°C. The bottles can be rolled at 1 rpm, but rolling is not necessary, and it may decrease cell viability.
5. Harvest the conditioned medium when the phenol red has turned orange, and clarify the medium by centrifugation at 200*g* for 10 min. Store the medium at 4°C.
6. Split the hybridomas at a ratio from 1:4 to 1:10, in fresh medium, to continue producing antibodies.

3. PURIFICATION OF MONOCLONAL ANTIBODIES (mABs)

For some applications, such as immunoadsorption and Western blotting, pure mAbs are not necessary, and hybridoma-conditioned medium or diluted ascites fluid may suffice. However, pure antibodies are required for applications such as immunohistochemistry and the determination of antibody affinity for antigen. There is no single method that allows for the purification of all species of mAbs. The most convenient method of purifying murine mAbs of immunoglobulin G (IgG) isotypes is chromatography on immobilized protein A (Ey et al., 1978) or protein G (Akerstrom et al., 1985). This method both concentrates and purifies the antibodies in a single step. However, this method cannot be used to purify IgM antibodies, and the low-pH buffers used to elute bound antibodies may have a deleterious effect on the activity of some antibodies.

3.1. Materials

Protein A-agarose (Pierce, cat. no. 20333) or protein G-agarose (Pierce, cat. no. 20398) slurry.
Phosphate-buffered saline (PBS), Ca^{2+}–Mg^{2+}-free.
Wash buffer: PBS, pH 7.4; or 3 *M* NaCl/50 m*M* Tris-HCl (pH 8.9) (High-salt buffer).
Elution buffer: 0.1 *M* glycine-HCl (pH 2.5).
Neutralization buffer: 1 *M* Tris-HCl (pH 7.5).
Columns (Bio-Rad, cat. no. 731-1550), 10-mL, disposable polypropylene.
Dialysis membrane with a mol wt cutoff of less than 100 kDa (Slide-A-Lyzer dialysis casette, Pierce, cat. no. 66407).

3.2. Procedure

1. Add 1–5 mL protein A- or protein G-agarose slurry to a polypropylene column. Wash the column with 5 column volumes of elution buffer, followed by 5 column volumes of wash buffer. Use high-salt buffer, if purifying IgG_1 on protein A (*see* Section 3.1. above).
2. Run the clarified antibody supernatant (conditioned medium) over the column, using gravity feed or a peristaltic pump at a flow rate of 1 mL/min.

3. Wash the column with 50–100 column volumes of wash buffer. High-salt buffer is only necessary if washing IgG_1 bound to protein A.
4. Elute bound antibody in 1-mL fractions, with at least 5 column volumes of elution buffer.
5. Add 0.2 mL 1 *M* Tris-HCl (pH 7.5), to neutralize each fraction of eluate.
6. Pool fractions containing antibodies, as determined by absorbance at 280 nm, a serological assay, or sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Dialyze the pooled antibodies against PBS or distilled water.
7. Determine the antibody concentration spectrophotometrically by absorbance at 280 nm (1 mg/mL IgG = 1.4 OD_{280} with a 1-cm light path).
8. Monitor the purity of the antibody preparation by SDS-PAGE. The heavy and light chains of reduced IgG run as 50 and 25 kDa bands, respectively.

3.3. Comments

1. Murine IgG_1 binds poorly to protein A, but this interaction can be substantially improved by adding NaCl to the antibody solution, to a final concentration of 3 *M*, and by raising the pH of the antibody solution to 8.9. Alternatively, murine IgG_1, binds with high affinity to protein G at physiological salt concentrations.
2. If the antigen-binding activity of the antibody of interest is adversely affected by the low-pH buffer used to elute antibodies from protein A or protein G, it may be possible to raise the pH of the elution buffer by up to 1 pH unit or more to reduce this effect, while still recovering antibody from the column. If this method is unsatisfactory, chromatography on a solid support, coupled to diethylaminoethyl (DEAE) and the dye, Cibacron blue F3GA (DEAE-Affigel Blue, Bio-Rad), can be used to purify all IgG isotypes at a pH closer to 7.4 (Bruck et al., 1982). The drawbacks of this method are:
   a. Antibodies do not bind to this matrix in 0.15 *M* NaCl, and therefore they are not concentrated.
   b. The final antibody preparation will contain transferrin.
3. IgM mAbs cannot be purified on protein A or protein G. However, IgM precipitates in low-ionic-strength solvent, so that can be purified from conditioned medium by dialysis against distilled water (Heide and Schwick, 1978). The precipitate is collected by centrifugation and redissolved in PBS. If necessary, IgM can be further purified by gel filtration chromatography on Sephadex G-200 or similar gel filtration medium.

4. VERIFYING ANTIBODY ACTIVITY

Once a mAb has been purified, it is important to verify that it has retained its antigen-binding activity, before it is used in further experiments. The most convenient methods for assessing antigen-binding activity are Western blotting using purified target antigen or a complex cell or tissue fraction, and immunoadsorption coupled with SDS-PAGE. A protocol for immunoadsorption is provided below.

4.1. Materials

Protein sample containing the target antigen.
mAb solution.
Protein A-agarose or protein G-agarose slurry.
Immunoprecipitation buffer.
3X SDS-PAGE sample buffer (reducing).
Eppendorf tubes, 1.5-mL.

Microcentrifuge.
Minigel apparatus.

4.2. Immunoadsorption

1. Solubilize antigen in 50–100 µL immunoprecipitation buffer in a 1.5-mL Eppendorf tube. Centrifuge the solution at 13,000*g* in a microcentrifuge, and transfer the supernatant to a second tube.
2. Add 1–10 µg mAb, and incubate the antigen-antibody mixture at 4°C for at least 1 h.
3. Add 50 µL protein A-agarose or protein G-agarose in immunoprecipitation buffer, and incubate at 4°C for 1–2 h.
4. Pellet the beads by centrifugation at 13,000*g* in a microcentrifuge, and wash the antibody-antigen complexes, adsorbed to the protein A or protein G beads, with 0.5 mL immunoprecipitation buffer, 5×. Collect the beads by centrifugation after each wash.
5. Wash the beads a final time with distilled water, to reduce the amount of salt in the sample.
6. Add an appropriate volume of 1X SDS-PAGE sample buffer, heat the sample for 5 min at 95°C, then put it on ice.
7. Electrophorese the sample in an SDS-polyacrylamide minigel of appropriate concentration. Stain the gel with Coomassie brilliant blue or silver, to detect the protein antigen.

4.3. Comments

1. In Western blotting, if epitope reactivity is sensitive to reducing agents, omit 2-mercaptoethanol from the electrophoresis sample buffer.
2. mAbs raised against native protein antigens may not be suitable for use in Western blotting. Immunoadsorption is likely to be a more suitable application for these antibodies. Conversely, mAbs raised against denatured protein antigens may be most suited for use in Western blotting.
3. Murine mAbs that do not bind protein A or protein G can be used in immunoadsorption assays, by precoating the protein A or protein G beads with secondary polyclonal antibodies such as rabbit antimouse Ig. If this method is used, it is important to include a negative control omitting the mAb.
4. Complex protein antigens such as cell or tissue lysates, may be preferable to pure antigen in Western blotting or immunoadsorption assays of mAbs, because they will provide additional information about antibody crossreactivities.
5. An additional method by which mAbs can be verified is antibody typing. By identifying antibody heavy and light chains, this assay provides supporting evidence that the antibody that has been purified is indeed correct. Enzyme-linked immunosorbant assay-based typing kits are available from a number of commercial sources.

5. APPENDIX

5.1. Media and Supplements

1. RD nutrient medium (a 1:1 mixture of RPMI-1640 and Dulbecco's modified Eagle's medium):
   a. Dulbecco's modified Eagle's medium with high glucose (Gibco-BRL, cat. no. 12100-046). Formulation:

Inorganic Salts:	mg/L
$CaCl_2$ (anhydrous)	200.00
$Fe(NO_3) \cdot 9H_2O$	0.10

KCl	400.00
$MgSO_4$ (anhydrous)	97.67
NaCl	6400.00
$NaH_2PO_4 \cdot H_2O$	125.00
Other Components:	
D-Glucose	4500.00
Phenol red	15.00
Amino Acids:	
L-Arginine · HCl	84.00
L-Cysteine 2HCl	63.00
L-Glutamine	584.00
Glycine	30.00
L-Histidine HCl · H_2O	42.00
L-Isoleucine	105.00
L-Leucine	105.00
L-Lysine HCl	146.00
L-Methionine	30.00
L-Phenylalanine	66.00
L-Serine	42.00
L-Threonine	95.00
L-Tryptophan	16.00
L-Tyrosine 2Na · $2H_2O$	104.00
L-Valine	94.00
Vitamins:	
D-Calcium pantothenate	4.00
Choline chloride	4.00
Folic acid	4.00
i-Inositol	7.20
Niacinamide	4.00
Riboflavin	0.40
Thiamine HCl	4.00
Pyridoxine HCl	4.00

b. RPMI-1640 formulation:

Inorganic salts	mg/L
$Ca(NO_3)_2 \cdot 4H_2O$	100.00
KCl	400.00
$MgSO_4$ (anhyd.)	48.84
NaCl	6000.00
Na_2HPO_4 (anhyd.)	800.00
Other Components:	
D-Glucose	2000.00
Glutathione (reduced)	1.00
Phenol red	5.00
Amino Acids:	
L-Arginine	200.00
L-Asparagine (free base)	50.00
L-Aspartic acid	20.00
L-Cystine 2HCl	65.00
L-Glutamic acid	20.00

L-Glutamine	300.00
Glycine	10.00
L-Histidine (free base)	15.00
L-Hydroxyproline	20.00
L-Isoleucine	50.00
L-Leucine	50.00
L-Lysine · HCl	40.00
L-Methionine	15.00
L-Phenylalanine	15.00
L-Proline	20.00
L-Serine	30.00
L-Threonine	20.00
L-Tryptophan	5.00
L-Tyrosine 2Na · $2H_2O$	29.00
L-Valine	20.00
Vitamins:	
Biotin	0.20
D-Calcium pantothenate	0.25
Choline chloride	3.00
Folic acid	1.00
I-Inositol	35.00
Niacinamide	1.00
Para-aminobenzoic acid	1.00
Pyridoxine HCl	1.00
Riboflavin	0.20
Thiamine HCl	1.00
Vitamin B_{12}	0.005

c. Reconstitute equivalent amounts of powdered RPMI 1640 (Gibco-BRL, cat. no. 31800-022) and DMEM with high glucose with Milli-Q water according to the manufacturer's instructions to give a final 1:1 mixture (by volume).

d. Stir in the following reagents to the final concentrations:

Component	Final concentration
HEPES (Research Organics, Cleveland, OH, cat no. 6003H)	15 m*M*
L-Glutamine (Sigma, cat. no. G-3126)	2 m*M*
Na pyruvate (Sigma, cat. no. P-2256)	0.01%
$NaHCO_3$ (J.T. Baker, cat. no. 3506-01)	2.0 g/L
Penicillin (Sigma, cat. no. P-3032)	100 IU/mL
Streptomycin (Sigma, cat. no. P-3032)	50 µg/mL

e. Adjust the pH to 7.5, and sterilize the medium by filtration through a 0.22-µm filter.

2. Medium supplements, 200X stock solutions:

a. Make the following stock solutions:

i. Insulin (Sigma, cat. no. I-5500), 200X stock.
2 mg/mL insulin in 10 m*M* HCl.

ii. Transferrin (Sigma, cat. no. T-2252), 200X stock.
2 mg/mL human transferrin in PBS.

iii. Ethanolamine (Sigma, cat. no. E-9508), 200X stock.

2 m*M* ethanolamine in Milli-Q water.

iv. 2-Mercaptoethanol (Sigma, cat. no. M-6250), 200X.
2 m*M* 2-mercaptoethanol in Milli-Q water.

v. Na selenite (Sigma, cat. no. S-1382), 200X.
2 μ*M* Na selenite in Milli-Q water.

b. Sterilize stock solutions by filtration through 0.22-μm filters.

c. Keep supplements at –20°C for long-term storage.

3. Fatty acid-free bovine serum albumin (BSA) (Bayer, cat. no. 82-002-2) conjugated with oleic acid (Sigma, cat. no. O-1008) (50X Stock solution).
   a. Make a 50-mg/mL solution of fatty acid-free Fraction V BSA in PBS. Filter-sterilize through 0.22-μm filter.
   b. Make a 20-mg/mL solution of oleic acid in 100% ethanol.
   c. For each mL BSA solution, add, dropwise, with constant stirring at 37°C, 25 μL 20 mg/mL solution of oleic acid (final concentration 500 μg/mL). If the solution is turbid, incubate it in a 37°C water bath until it clears.

   Note: A commercial BSA–oleic acid conjugate is available from Sigma (cat. no. O-3008).

   d. Store the 50X stock solution at 4°C in a light-tight container.

4. RD+5F (Factor) medium:
   a. Add appropriate volumes of the 200X stock solutions of insulin, transferrin, ethanolamine, 2-mercaptoethanol, and Na selenite to RD nutrient medium.
   b. Supplement the medium with BSA–oleic acid to final concentrations of 1 mg/mL BSA, which corresponds to 10 μg/mL oleic acid.

5.2. PBS, Ca^{2+}-Mg^{2+}-Free

NaCl	8.00 g/L
KCl	0.20 g/L
KH_2PO_4	0.20 g/L
$NaHPO_4$	1.15 g/L

Adjust pH to 7.4.

5.3. Immunoadsorption Buffers

1. Immunoprecipitation buffer:
   PBS (*see* 5.2), adjusted to 0.5 *M* NaCl (pH 7.4).
   0.1% SDS (Sigma, cat. no. L-4509).
   1% NP-40 (Sigma, cat. no. N-6507).
   0.5% Na deoxycholate (Sigma, cat. no. D-6750).
2. 3X SDS-PAGE sample buffer (reducing):

0.5 *M* Tris-HCl (pH 6.8)/0.4% SDS	1 mL
25% SDS in H_2O	0.8 mL
2-Mercaptoethanol (Sigma, cat. no. M-6250)	0.5 mL
Glycerol (J.T. Baker, cat. no. 2140-01)	1 mL
Bromophenol blue (Bio-Rad, cat. no. 161-0404)	0.05%

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FURTHER READING

Akerstrom, B., Brodin, T., Reis, K., and Bjorck, L. (1985), Protein G: a powerful tool for binding and detection of monoclonal and polyclonal antibodies. *J. Immunol.* **135**, 2589–2592.

Bruck, C., Portetelle, D., Glineur, C., and Bollen, A. (1982), One-step purification of mouse monoclonal antibodies from ascitic fluid by DEAE Affi-Gel blue chromatography. *J. Immunol. Methods* **53**, 313–319.

Ey, P. L., Prowse, S. J., and Jenkin, C. R. (1978), Isolation of pure IgG_1, IgG_{2a}, and IgG_{2b} immunoglobulins from mouse serum using protein A-sepharose. *Immunochemistry* **15**, 429–436.

Heide, K. and Schwick, H. G. (1978), Salt fractionation of immunoglobulins, in: *Handbook of Experimental Immunology*, vol. 1, 3rd ed., Weir, D. M., ed., Blackwell, Oxford, UK, pp. 7.1–7.11.

Kawamoto, T., Sato, J. D., McClure, D. B., and Sato, G. H. (1986), Serum-free medium for the growth of NS-1 mouse myeloma cells and the isolation of NS-1 hybridomas. *Methods Enzymol.* **121**, 266–277.

Myoken, Y., Okamoto, T., Osaki, T., Yabumoto, M., Sato, G. H., Takada, K., and Sato, J. D. (1989), An alternative method for the isolation of NS-1 hybridomas using cholesterol auxotrophy of NS-1 mouse myeloma cells. *In Vitro Cell. Dev. Biol.* **25**, 477–480.

Sato, J. D., Kawamoto, T., and Okamoto, T. (1987), Cholesterol requriement of P3-X63-Ag8 and X63-Ag8.653 myeloma cells for growth *in vitro*. *J. Exp. Med.* **165**, 1761–1766.

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